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1 **One week of step reduction lowers myofibrillar protein synthesis rates in young men**

2

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16

17 **Running title:** Step reduction and muscle protein synthesis

18

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26 **ABSTRACT**

27 **PURPOSE:** Across the lifespan, physical activity levels decrease and time spent sedentary
28 typically increases. However, little is known about the impact that these behavioural changes
29 have on skeletal muscle mass regulation. The primary aim of this study was to use a step
30 reduction model to determine the impact of reduced physical activity and increased sedentary
31 time on daily myofibrillar protein synthesis rates in healthy young men. **METHODS:** Eleven
32 men (22 ± 2 y) completed 7 days of habitual physical activity (HPA) followed by 7 days of
33 step reduction (SR). Myofibrillar protein synthesis rates were determined during HPA and SR
34 using the deuterated water ($^2\text{H}_2\text{O}$) method combined with the collection of skeletal muscle
35 biopsies and daily saliva samples. Gene expression of selected proteins related to muscle
36 mass regulation and oxidative metabolism were determined via real time RT-qPCR.

37 **RESULTS:** Daily step count was reduced by approximately 91% during SR (from
38 13054 ± 2763 to 1192 ± 330 steps $\cdot\text{d}^{-1}$; $P<0.001$) and this led to an increased contribution of
39 sedentary time to daily activity (73 ± 6 to $90\pm 3\%$; $P<0.001$). Daily myofibrillar protein
40 synthesis decreased by approximately 27% from 1.39 ± 0.32 % $\cdot\text{d}^{-1}$ during HPA to 1.01 ± 0.38
41 % $\cdot\text{d}^{-1}$ during SR ($P<0.05$). MAFbx and myostatin mRNA expression were up-regulated
42 whereas mTOR, p53 and PDK4 mRNA expression were down-regulated following SR
43 ($P<0.05$). **CONCLUSION:** One week of reduced physical activity and increased sedentary
44 time substantially lowers daily myofibrillar protein synthesis rates in healthy young men.

45 **KEY WORDS:** Skeletal muscle, physical activity, inactivity, sedentary.

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50 INTRODUCTION

51 Skeletal muscle mass, physical function and metabolic health progressively decline with
52 advancing age. This could be attributed to the gradual reduction in levels of physical activity
53 and/or the increase in sedentary behaviour that typically occurs across the lifespan (1). The
54 importance of physical activity in maintaining skeletal muscle mass and function is well
55 appreciated and recent evidence has implicated sedentariness, distinct from physical activity,
56 as a risk factor for age-related loss of skeletal muscle mass and strength (2). However, the
57 physiological processes which may contribute to the negative consequences of physical
58 inactivity and sedentary behaviour on skeletal muscle mass and function are relatively
59 unknown.

60 Skeletal muscle mass is governed by overall protein balance, which is determined by rates of
61 muscle protein synthesis and breakdown. Any loss of muscle mass must be explained by an
62 overall negative protein balance (i.e., muscle protein breakdown must exceed muscle protein
63 synthesis). Extreme muscle disuse (i.e., bed rest or limb immobilization), where voluntary
64 muscular contractile activity is essentially removed, results in substantial loss of skeletal
65 muscle mass (3, 4). This is associated with reductions in postabsorptive muscle protein
66 synthesis rates and the development of 'anabolic resistance'; that is, a reduced stimulation of
67 postprandial muscle protein synthesis (3). On the other hand, the impact of disuse on muscle
68 protein breakdown rates in humans is poorly defined. Whilst disuse models provide
69 invaluable information on the impact of severe muscle unloading, their extreme nature may
70 not accurately reflect typical physically inactive and sedentary lifestyles (5).

71 Step reduction has been proposed as a model to more accurately examine the underlying
72 physiology of physically inactive and sedentary individuals and also explore the
73 physiological changes that occur when inactivity is enforced by injury, illness and/or other

74 significant life events (5-7). Step reduction has been shown to reduce insulin sensitivity (8,
75 9), but few studies have evaluated its impact on skeletal muscle mass regulation. Breen and
76 colleagues reported reduced postprandial myofibrillar protein synthesis rates following 14
77 days of step reduction in older, overweight individuals (10). These findings are supported by
78 more recent data showing reduced integrated myofibrillar protein synthesis rates throughout a
79 two-week period of step reduction in older, overweight adults (11).

80 While these findings are important, there are currently no data available on muscle protein
81 synthesis rates over shorter periods of time typically associated with recovery from injury
82 and/or acute illness (i.e., \leq one week). The above studies also describe responses in older,
83 overweight individuals, but young and older individuals have distinct responses to muscle
84 disuse (12, 13). The influence of step reduction on skeletal muscle mass regulation in young
85 individuals has yet to be studied but is important to characterize to enhance our understanding
86 of the impact of reduced physical activity and increased sedentary behaviour on skeletal
87 muscle mass regulation at various stages across the lifespan.

88 Accordingly, the primary purpose of the present study was to use the step reduction model to
89 determine the impact of short-term reduced physical activity and increased sedentary time on
90 myofibrillar protein synthesis rates in healthy young men. The deuterated water ($^2\text{H}_2\text{O}$)
91 approach was used as it allows myofibrillar protein synthesis rates to be measured under free-
92 living conditions over time frames where quantifiable changes in muscle mass are unlikely to
93 occur, providing important insight into longer-term muscle mass regulation (14-16). It was
94 hypothesized that one week of reduced physical activity and increased sedentary time would
95 reduce daily myofibrillar protein synthesis rates.

96 **METHODS**

97 *Participants and ethical approval*

98 Eleven healthy young men participated in the present study which took place between June
99 2016 and February 2018. All participants were recreationally active and self-reported
100 engaging in structured physical activity ≥ 3 times/week for > 6 months prior to inclusion.
101 Five of the participants reported undertaking only aerobic-based exercise, four of the
102 participants reported undertaking only resistance-based exercise and two of the participants
103 reported undertaking both aerobic and resistance-based exercise. None of the participants
104 were competitive endurance and/or power athletes. The participants' baseline characteristics
105 are presented in **Table 1**. Prior to providing informed written consent, each volunteer was
106 informed of the experimental procedures and potential risks associated with the experimental
107 intervention. Participants were screened prior to inclusion in the study and deemed healthy
108 based on their responses to a general health questionnaire. Exclusion criteria included being a
109 current or recent (last 6 months) smoker, hypertensive ($\geq 140/90$ mmHg), diagnosed with
110 diabetes and/or suffering from a recent injury. Participants deemed eligible were
111 subsequently fitted with an ActivPAL3TM accelerometer (see *Accelerometry* section below)
112 for 7 days to objectively assess daily step count. Any individual completing <7000 steps \cdot d⁻¹
113 was excluded from participating in the study. The study was approved by the National
114 Research Ethics Service Committee West Midlands, Edgbaston, United Kingdom (Reference:
115 16/WM/0011) and conformed to standards for the use of human participants in research as
116 outlined in the Declaration of Helsinki. The intervention was registered at clinicaltrials.gov
117 prior to data collection (Identifier: NCT02624011).

118 *Study overview*

119 An overview of the study is presented in **Figure 1**. Following an initial ²H₂O dosing day (day
120 -2) and one maintenance day (see *²H₂O dosing protocol* section below), participants
121 completed 7 days of habitual physical activity (HPA) followed by 7 days of step reduction
122 (SR). For the first 7 days, participants were instructed to maintain their habitual physical

123 activity levels (i.e., regular ambulation and structured physical activity). During SR,
124 participants were instructed to reduce their step count to ~ 1500 steps $\cdot d^{-1}$, be as sedentary as
125 possible and refrain from any form of structured physical activity for the remaining 7 days. A
126 target daily step count of ~ 1500 steps $\cdot d^{-1}$ was set during SR as large-scale global data suggest
127 that the average daily step count for adults is < 5000 steps $\cdot d^{-1}$ (17). An ActivPAL3TM
128 accelerometer (see *Accelerometry* section below) was worn throughout HPA and SR to
129 objectively assess physical activity levels and sedentary time. As the ActivPAL3TM
130 accelerometer does not provide visual feedback on daily step count, participants were also
131 provided with a pedometer during the SR period to help prevent their daily step count
132 exceeding the 1500 steps $\cdot d^{-1}$ threshold. A member of the investigative team was on call
133 throughout the SR period to help participants with activities of daily living (e.g., food
134 shopping) that were not practical within the step count parameters set out. Weighed 4-day
135 food diaries were completed during HPA and SR (see *Dietary intake* section below). Muscle
136 biopsies were collected on days 0, 7 and 14, saliva samples were collected daily and an oral
137 glucose tolerance test (OGTT) was conducted on days 7 and 14.

138 *Experimental visits*

139 On the morning of day 0, participants arrived at the laboratory at 08:00 in a fasted state from
140 22:00 the evening before. After voiding, participants were weighed in light clothing to the
141 nearest 0.1 kg (Ohaus, Champ II scales, USA) and height measured to the nearest centimetre
142 (Stadiometer, Seca, UK). Body composition (whole-body fat-free mass and body fat
143 percentage) was subsequently determined by dual-energy X-ray absorptiometry (DXA,
144 Discovery QDR W series; Hologic). Following the DXA scan, a saliva sample (see ²H₂O
145 *dosing protocol* section below) was obtained before collection of a muscle biopsy from the
146 *vastus lateralis* muscle. Muscle biopsies were collected using the Bergström needle technique
147 with manual suction under local anaesthesia (1% lidocaine). Muscle biopsy samples were

148 blotted and any visible fat, blood or connective tissue removed before snap freezing in liquid
149 nitrogen and storing at -80°C for later analysis. Subsequent muscle biopsies (days 7 and 14)
150 were taken from separate incisions in an alternating pattern between legs. Participants then
151 consumed a single maintenance bolus of $^2\text{H}_2\text{O}$ (see *$^2\text{H}_2\text{O}$ dosing protocol* section below)
152 before being fitted with an ActivPAL3TM accelerometer prior to leaving the laboratory.

153 Following 7 days of HPA, participants returned to the laboratory at 08:00 on day 7, again in a
154 fasted state from 22:00 the evening before. Participants were weighed prior to insertion of a
155 20G cannula into an antecubital vein to allow for repeated blood sampling during the OGTT.
156 A saliva sample was subsequently obtained before collection of the second muscle biopsy.
157 Following the muscle biopsy, a baseline blood sample was then drawn before participants
158 completed an OGTT. Participants consumed 75 g dextrose as a 25% solution with subsequent
159 blood samples drawn at 30, 60, 90 and 120 minutes to assess postprandial blood glucose,
160 insulin and non-esterified fatty acid (NEFA) concentration responses. Blood samples were
161 collected into EDTA-containing Vacutainers (BD, New Jersey, USA) prior to centrifugation
162 at 1500 g for 15 minutes at 4°C . Aliquots containing plasma were stored at -80°C .
163 Participants remained in a semi-supine position throughout the OGTT and once completed,
164 consumed a single maintenance bolus of $^2\text{H}_2\text{O}$ before leaving the laboratory. Following 7
165 days of SR, participants arrived at 08:00 in a fasted state for the final laboratory visit (i.e.,
166 day 14) which was identical to the experimental protocol completed on day 7.

167 *$^2\text{H}_2\text{O}$ dosing protocol*

168 The $^2\text{H}_2\text{O}$ dosing protocol consisted of one dosing day and 16 maintenance days (14). On day
169 -2, participants completed a $^2\text{H}_2\text{O}$ loading day. Following collection of a background saliva
170 sample, participants were provided with 8 x 50 mL boluses of 70% $^2\text{H}_2\text{O}$ (Cambridge Isotope
171 Laboratories, Massachusetts, USA) to increase deuterium (^2H) enrichment in body water to

172 0.5-1%. Approximately 60-90 minutes was allowed between each bolus to negate side effects
173 (e.g., vertigo, nausea) previously reported upon consumption of large volumes of $^2\text{H}_2\text{O}$ over
174 short periods of time. The $^2\text{H}_2\text{O}$ protocol was well tolerated with none of the participants
175 reporting any adverse effects. For each subsequent day, participants were provided with a
176 daily 50 mL maintenance bolus of $^2\text{H}_2\text{O}$ to consume. Participants were instructed to consume
177 the daily bolus upon waking up to ensure consistency and minimize the risk of missed doses.
178 The time at which each bolus was consumed was recorded and participants were instructed to
179 bring the empty bottles back in on each laboratory visit to measure compliance. All boluses
180 were returned void, suggesting full compliance with the $^2\text{H}_2\text{O}$ protocol.

181 To measure ^2H enrichment in body water, saliva samples were collected daily. Participants
182 lightly chewed a cotton swab until completely saturated with saliva (~2-3 minutes). On days -
183 2, 0, 7 and 14, swabs were collected in the laboratory, immediately placed in a 5 mL syringe
184 and the saliva compressed into sample tubes and stored at -80°C for later analysis. On the
185 remaining days when participants were not in the laboratory, daily saliva samples were
186 collected at home and stored in pre-labelled falcon tubes in the fridge until the next
187 laboratory visit where samples were stored as described above. Participants were instructed to
188 provide their saliva sample at least 2 hours following their last $^2\text{H}_2\text{O}$ bolus and at least 30
189 minutes after their last meal or drink and to record the time at which the sample was
190 collected.

191 *Accelerometry*

192 During the screening process, participants were fitted with an ActivPAL3TM accelerometer
193 (PAL Technologies Ltd., Glasgow, UK) to assess daily step count. Participants were also
194 fitted with an ActivPAL3TM accelerometer during HPA and SR to objectively assess physical
195 activity levels and sedentary time. The ActivPAL3TM accelerometer was attached to the

216 anterior of the upper thigh using waterproof dressing. Participants were required to wear the
217 accelerometer at all times except when bathing. Complete 14-day accelerometry data were
218 obtained from all 11 participants over the experimental intervention. During the 7-day period
219 of SR, participants were also provided with a hip-worn pedometer (Yamax Digi-Walker SW-
220 200) which provided visual feedback on their step count to aid compliance with the 1500
221 steps·d⁻¹ requirement. Daily step count from the hip-worn pedometer was recorded by
222 participants before bed. Accelerometry data were downloaded from devices using
223 ActivPAL3™ analysis software (PAL Technologies Ltd., Glasgow, UK, v7.2.32).

224 *Dietary intake*

225 The evening prior to each experimental visit on days 0, 7 and 14, participants received the
226 same standardized meal (~689 kcal, providing ~55 energy% (En%) carbohydrate, ~20 En%
227 protein, and ~25 En% fat). A weighed 4-day food diary was completed over the first 7-day
228 period of HPA and over the second 7-day period of SR to evaluate energy and macronutrient
229 intake. Participants were required to include two week-days and both weekend days in their
230 recordings. Dietary records were analysed using Dietplan software (Forestfield Software Ltd.,
231 v6.70.67).

232 *Plasma analyses*

233 Plasma glucose (Glucose Oxidase kit, Instrumentation Laboratories, Cheshire, UK) and
234 NEFA (NEFA kit, Randox, London, UK) concentrations were analysed in duplicate using
235 enzymatic colorimetric assays using an ILAB 650 Clinical Chemistry Analyser
236 (Instrumentation Laboratory, Warrington, UK). Plasma insulin concentrations were
237 determined in duplicate using commercially available enzyme-linked immunosorbent assay
238 (ELISA) kits (Invitrogen, California, United States, KAQ1251).

219 *Body water ²H enrichment*

220 Body water ²H enrichment was analysed from daily saliva samples collected throughout the
221 study as previously described (14). Briefly, samples were centrifuged at 10000 g and then
222 diluted 70-fold with ddH₂O. Subsequently, small plastic cups holding 4 mg of catalyst (5 %
223 platinum on alumina, 325 mesh, Sigma-Aldrich, St. Louis, USA) were placed inside 3 mL
224 glass vials (Labco Exetainer, Labco limited, Lampeter, UK) and 300 µL of diluted saliva was
225 then added. Air in each vial was simultaneously evacuated and replaced by hydrogen gas.
226 Once prepared, the vials were left at 21 °C for 24 hours for ²H equilibration to occur between
227 the hydrogen gas and the saliva samples. The ²H enrichment of the hydrogen gas was then
228 measured in duplicate on a GC-C-IRMS (Micromass 205 Optima IRMS fitted with a
229 Multiprep and Gilson autoinjector, Micromass UK Limited, 206 Manchester, UK). Standard
230 regression curves were applied to assess the linearity of the mass spectrometer and to account
231 for ²H loss during equilibration.

232 *Myofibrillar bound ²H-alanine enrichment*

233 For measurement of ²H-alanine enrichment in the myofibrillar fractions, ~50 mg wet muscle
234 tissue was hand-homogenized on ice using a pestle in a standard extraction buffer (10 µL·mg⁻¹).
235 The samples were then spun at 2500 g for 5 minutes at 4°C. The pellet was washed with
236 500 µL of ddH₂O and centrifuged at 250 g for 10 minutes at 4°C. The myofibrillar protein
237 was solubilized by adding 1 mL of 0.3 M NaOH and heating at 50°C for 30 minutes with
238 vortex mixing every 10 minutes. Samples were centrifuged at 9500 g for 5 minutes at 4°C,
239 the supernatant containing the myofibrillar proteins was collected and the collagen pellet was
240 discarded. Myofibrillar proteins were precipitated by the addition of 1 mL of 1 M PCA and
241 spinning at 700 g for 10 minutes at 4 °C. The myofibrillar protein was washed twice with
242 70% ethanol and hydrolysed overnight in 2 mL of 6 M HCL at 110°C. The free amino acids

243 from the hydrolysed myofibrillar protein pellet were dried under a nitrogen stream while
244 being heated to 120 °C. The free amino acids were then dissolved in 25% acetic acid solution,
245 passed over cation exchange AG 50W-X8 resin columns (mesh size: 100-200, ionic form:
246 hydrogen; Bio-Rad Laboratories, Hercules, CA), and eluted with 2 M NH₄OH. Thereafter,
247 the eluate was dried, and the purified amino acids were derivatized to their N(O,S)-
248 ethoxycarbonyl ethyl esters. The derivatized samples were measured using a gas
249 chromatography-isotope ratio mass spectrometer (GC-IRMS) (Thermo Fisher Scientific,
250 MAT 253; Bremen, Germany) equipped with a pyrolysis oven and a 60 m DB-17MS column
251 (no. 122-4762; Agilent, Wilmington, DE, USA) and 5 m precolumn. Ion masses 2 and 3 were
252 monitored to determine the ²H/¹H ratios of myofibrillar protein bound alanine. A series of
253 known standards were applied to assess linearity of the mass spectrometer and to control for
254 the loss of tracer.

255 *Gene expression analysis*

256 Total RNA was isolated from ~20 mg of frozen powdered muscle tissue by homogenising in
257 1 mL of TRI Reagent (Sigma Aldrich, Gillingham, UK) using an IKA T10 basic ULTRA-
258 TURRAX homogenizer (IKA, Oxford, UK). To achieve phase separation, 200 µL of
259 chloroform was added to each sample followed by vigorous shaking for 15 seconds, 15
260 minutes at ambient temperature and subsequent centrifugation at 12000 g for 15 minutes at
261 4°C. The RNA-containing supernatant was then removed and mixed with an equal volume of
262 2-propanol. RNA was purified on Reliaprep spin columns (Promega, Madison, Wisconsin,
263 USA) using the manufacturer's instructions, which includes a DNase treatment step. A
264 FLUOstar Omega microplate reader (LVis function) was used to determine the RNA
265 concentration and purity of each sample. The ratio of absorbance at 260 nm and 280 nm was
266 ≥ 2.0 for all samples. 900 ng of total RNA was reverse-transcribed to cDNA in 20 µL
267 volumes using the nanoScript 2 RT kit and a combination of oligo(dT) and random primers

268 (Primerdesign, Southampton, UK) as per the manufacturer's instructions. The resultant
269 cDNA was diluted to 10 ng/mL prior to RT-qPCR analysis. All analysis was performed in
270 triplicate using Primerdesign custom designed primers (Supplementary Table 1) or
271 commercially available 18S, GAPDH, TOP1, B2M and ACTB (Primerdesign Southampton,
272 UK) and Precision plus qPCR Mastermix with low ROX and SYBR (Primerdesign
273 Southampton, UK) on a QuantStudio3 Real-Time PCR System (Applied Biosystems, Thermo
274 Fisher, UK). Dependent on the gene of interest, 10-50 ng of cDNA was added to each well in
275 a 20 uL reaction volume. Thermal cycling conditions were 2 minutes at 95°C and 40 cycles
276 of 10 seconds at 95°C and 60 seconds at 60°C. A post qPCR run melt curve (Applied
277 Biosystems, Thermo Fisher, UK) was used to ascertain the specificity of each primer. qPCR
278 results were analysed using Experiment Manager (Thermo Fisher). mRNA expression values
279 are expressed as fold change relative to the average baseline (i.e., HPA) Δ CQ value using the
280 $2^{-\Delta\Delta CQ}$ method (18). To control for RNA input, the geometric mean of the CQ values for
281 TOP1, B2M and ACTB was used as an internal control as these were found to be the three
282 most stable genes across all samples using RefFinder (RefFinder, RRID:SCR_000472) (19).
283 All gene expression data are presented for n=10 as insufficient muscle tissue was available
284 for RNA isolation for one participant. Statistical analysis was performed on the $2^{-\Delta\Delta CQ}$
285 transformed data.

286 *Calculations*

287 Total area under the curve (AUC) for plasma glucose and insulin concentrations was
288 calculated using the trapezoidal method. The Matsuda index, an index of whole-body insulin
289 sensitivity, was calculated as previously described (20). Myofibrillar protein fractional
290 synthetic rate (FSR) was determined using the incorporation of ^2H -alanine into myofibrillar
291 protein and the mean ^2H enrichment in body water between sequential biopsies, corrected by

292 a factor of 3.7, as the surrogate precursor based upon ²H labelling during *de novo* alanine
293 synthesis (14, 15). The standard precursor-product method was used to calculate FSR:

$$FSR (\% \cdot day^{-1}) = \left(\frac{E_{m2} - E_{m1}}{E_{precursor} \times t} \right) \times 100$$

294 where E_{m1} and E_{m2} are the myofibrillar protein-bound ²H-alanine enrichments between
295 sequential muscle biopsies. E_{precursor} represents the mean body water ²H enrichment between
296 sequential biopsies corrected by a factor of 3.7 based upon the ²H labelling of alanine during
297 *de novo* synthesis (14). *t* represents the time between sequential biopsies in days.

298 *Statistics*

299 Based on previous research (15), sample size calculations showed that n=9 would be
300 sufficient to detect a difference in daily myofibrillar protein synthesis rates between HPA and
301 SR conditions using a two-tailed paired samples t-test (95% power, α-level of 0.05, G*power
302 version 3.1.9.2). Allowing for a 20% dropout rate, eleven participants were recruited. All
303 statistical analyses were performed using SPSS 22.0 (SPSS, RRID:SCR_002865, Chicago,
304 IL, USA). Differences between conditions (HPA vs. SR) for accelerometry, dietary intake,
305 plasma insulin and glucose AUC, Matsuda index, myofibrillar protein FSR and gene
306 expression were compared using paired sample t-tests. Body water ²H enrichment was
307 analysed using a one-factor repeated measures ANOVA with time as the within-subjects
308 factor. A two-factor repeated measures ANOVA (condition x time) with condition (HPA vs.
309 SR) and time (0, 30, 60, 90 and 120 minutes) as within-subjects factors was performed for
310 analysis of plasma glucose, insulin and NEFA concentrations. Bonferroni post-hoc tests were
311 conducted to correct for multiple comparisons when a significant condition x time interaction
312 was identified. All data are presented as mean±SD.

313 **RESULTS**

314 *Accelerometry*

315 Daily step count was reduced by approximately 91% during SR (13054 ± 2763 to 1192 ± 330
316 $\text{steps} \cdot \text{d}^{-1}$; $P < 0.001$). Self-reported pedometer-derived daily step count during SR (1312 ± 297
317 $\text{steps} \cdot \text{d}^{-1}$) was highly correlated with accelerometer-derived daily step count ($r = 0.851$;
318 $P = 0.001$). The percentage of total time spent sedentary (73 ± 6 to $90 \pm 3\%$; $P < 0.001$) increased
319 and percentage of total time spent standing (17 ± 6 to $8 \pm 3\%$; $P < 0.001$) and ambulatory
320 (10.0 ± 1.0 to $1.0 \pm 0.5\%$; $P < 0.001$) decreased during SR. The number of daily transitions from
321 a sitting to standing position was also significantly reduced during SR (46 ± 8 to 31 ± 10 ;
322 $P < 0.001$).

323 *Body weight and dietary intake*

324 Body weight was not different following HPA and SR (75.3 ± 11.0 to 75.1 ± 10.8 kg; $P > 0.05$).
325 Dietary intake during HPA and SR is presented in **Table 2**. Daily energy intake tended to
326 decrease ($P = 0.07$) whereas both daily protein intake ($P < 0.01$) and protein intake relative to
327 body weight ($P < 0.01$) significantly decreased during SR. However, absolute carbohydrate
328 and fat intake and the relative contribution of protein, carbohydrate and fat to overall energy
329 intake were unchanged across the intervention ($P > 0.05$).

330 *Oral glucose tolerance*

331 Following SR, fasting plasma glucose concentrations were unaltered ($P > 0.05$; **Figure 2A**),
332 whereas fasting plasma insulin concentrations increased ($P < 0.05$; **Figure 2C**). In response to
333 the OGTT, a significant main effect for time ($P < 0.001$) was observed, with plasma glucose
334 concentrations elevated at 30 minutes compared to all other time points ($P < 0.01$), at 60
335 minutes compared to baseline and 120 minutes ($P < 0.05$), and at 90 minutes compared to 120
336 minutes ($P < 0.05$; **Figure 2A**). Plasma glucose AUC (703 ± 118 to 788 ± 79 $\text{mmol} \cdot 120 \text{ min} \cdot \text{L}^{-1}$)

337 was not significantly altered by 7 days of SR ($P>0.05$; **Figure 2B**). In contrast, a significant
338 condition x time interaction ($P<0.01$) was observed for plasma insulin, with greater plasma
339 insulin concentrations at 60, 90 and 120 minutes of the OGTT following 7 days of SR
340 ($P<0.05$; **Figure 2C**). In line with these findings, plasma insulin AUC (4590 ± 1817 to
341 $6287\pm1363 \mu\text{IU}\cdot120 \text{ min}\cdot\text{mL}^{-1}$) was significantly greater following SR ($P<0.01$; **Figure 2D**),
342 corresponding with a decrease in the Matsuda index (6.5 ± 1.8 to 4.5 ± 0.7) ($P<0.01$; **Figure**
343 **2F**). A significant main effect for time ($P<.001$) was also observed for plasma NEFA
344 concentrations, with baseline values being significantly higher at baseline compared to all
345 other time points ($P<0.05$), at 30 minutes compared to 60, 90 and 120 minutes ($P<0.001$) and
346 at 60 minutes compared to 90 minutes ($P<0.05$; **Figure 2E**).

347 *Body water ^2H enrichment*

348 **Figure 3A** presents the mean body water ^2H enrichment on a day-by-day basis. Following the
349 loading phase on day -2 and a single maintenance day on day -1, body water ^2H enrichment
350 reached $0.54\pm0.09\%$ (day 0). Body water ^2H enrichment did not change significantly over the
351 duration of the study with an average body water ^2H enrichment of $0.59\pm0.12\%$ during HPA
352 and $0.64\pm0.17\%$ during SR ($P>0.05$).

353 *Myofibrillar protein synthesis*

354 As shown in **Figure 3B**, daily myofibrillar protein synthesis rates decreased by
355 approximately 27% from $1.39\pm0.32 \text{ \%}\cdot\text{d}^{-1}$ during HPA to $1.01\pm0.38 \text{ \%}\cdot\text{d}^{-1}$ during SR
356 ($P<0.05$).

357 *Gene expression*

358 The skeletal muscle mRNA expression of genes implicated in muscle mass regulation and
359 oxidative metabolism is presented in **Figure 4**. In relation to the regulation of muscle protein

360 synthesis, myostatin mRNA expression was increased following SR and this was paralleled
361 by reduced mTOR mRNA expression (both $P < 0.05$; **Figures 4C and 4D**). However, p70S6K
362 mRNA expression was unchanged following SR ($P > 0.05$; **Figure 4E**). In regards to muscle
363 protein breakdown, MuRF1 mRNA expression was unchanged ($P > 0.05$; **Figure 4A**), whereas
364 MAFbx mRNA expression was up-regulated following SR ($P < 0.05$; **Figure 4B**). p53 and
365 PDK4 mRNA expression both decreased following SR (both $P < 0.05$; **Figures 4F and 4G**)
366 with no change in PGC-1 α mRNA expression ($P > 0.05$; **Figure 4H**).

367 **DISCUSSION**

368 The major novel finding of the present study was that one week of reduced physical activity
369 and increased sedentary time led to a substantial (~27%) decline in daily myofibrillar protein
370 synthesis rates. This decline in myofibrillar protein synthesis was associated with increased
371 skeletal muscle mRNA expression of myostatin and MAFbx and decreased mRNA
372 expression of mTOR. The present findings also show that one week of reduced physical
373 activity and increased sedentary time led to a decline in whole-body insulin sensitivity, in
374 addition to decreasing skeletal muscle mRNA expression of selected genes related to
375 oxidative metabolism (i.e., PDK4 and p53). Together, these findings provide direct evidence
376 that reduced physical activity and increased sedentary time alters the physiological processes
377 which regulate skeletal muscle in healthy young individuals.

378 Across the lifespan, physical activity levels generally decrease and time spent sedentary
379 typically increases (1). Likewise, injury, illness and/or other significant life events often
380 necessitate short periods (typically 2-7 days) of reduced physical activity and increased
381 sedentariness (6, 7). The findings of the present study demonstrate for the first time that just
382 one week of reduced physical activity and increased sedentary time leads to significant
383 (~27%) declines in daily myofibrillar protein synthesis rates in young healthy individuals

384 **(Figure 3B)**. These findings extend previous observations of reduced postprandial and
385 integrated myofibrillar protein synthesis rates following two weeks of step reduction in older,
386 overweight adults (10, 11) and highlights the central role that day-to-day muscular contractile
387 activity plays in regulating muscle protein synthesis rates. Promotion of regular physical
388 activity and minimising sedentariness throughout the lifespan should be considered as
389 integral to the maintenance of skeletal muscle health.

390 The findings of McGlory and colleagues are most comparable as they also applied $^2\text{H}_2\text{O}$ to
391 measure daily myofibrillar protein synthesis rates (11). The ~27% decline in daily
392 myofibrillar protein synthesis observed in the present study is substantially greater than the
393 ~12% decline in integrated myofibrillar protein synthesis rates observed by McGlory and
394 colleagues (11). This may be related to the greater relative change in daily step count induced
395 by this step reduction intervention (~91%) compared to McGlory et al. (~70%) (11).

396 Alternatively, this discrepancy could be explained by the duration of step reduction or
397 differences in the populations studied (i.e., younger vs. older adults). For example, some, but
398 not all, human muscle disuse studies have shown that 5-14 days of limb immobilization
399 results in greater loss of muscle mass in younger individuals when compared to older
400 individuals (12, 21). Thus, it could be hypothesized that a similar pattern of response is seen
401 from the perspective of muscle protein synthesis, whereby younger individuals are more
402 susceptible to changes in physical activity status than older individuals. In this regard, an
403 older comparator group to directly assess age-related differences in the present study would
404 have been informative and is an important avenue for future research.

405 A number of factors including habitual physical activity (10), diet composition (22), energy
406 balance (23) and sleep (24) can influence day-to-day muscle protein synthesis rates. It is also
407 well established that dietary protein/amino acid administration robustly stimulates muscle
408 protein synthesis (25-27). Whilst dietary protein intake decreased from habitual levels during

409 SR, it is important to note that participants were still consuming 133 ± 13 g·d⁻¹ of dietary
410 protein during the SR period (**Table 2**). When expressed relative to body weight, this equates
411 to a protein intake of 1.8 ± 0.2 g·kg⁻¹·d⁻¹. This intake is well above the established
412 recommended dietary allowance for protein of 0.8 g·kg⁻¹ of body weight and is also greater
413 than recently proposed changes to those recommendations (i.e., 1.2 - 1.6 g·kg⁻¹ of body
414 weight) (28). Energy balance can also influence muscle protein synthesis rates, with studies
415 showing that energy restriction reduces myofibrillar protein synthesis rates in young and
416 older individuals (23, 29, 30). In the present study, daily energy intake tended ($P=0.07$) to
417 decrease during SR but body weight remained stable, suggesting that participants were not in
418 negative energy balance. Whilst it is unlikely that modifications in dietary protein and/or
419 energy intake contributed to the decline in daily myofibrillar protein synthesis rates during
420 one week of SR, these factors and other dietary related variables (e.g., protein distribution
421 across the day) cannot be completely ruled out and thus future studies should investigate the
422 independent and combined impact of these variables on muscle mass regulation.

423 Previous studies that employed stable isotope infusion protocols within a laboratory setting
424 provide some insight into what could explain the reduction in daily myofibrillar protein
425 synthesis rates observed herein. For example, the reduction in regular muscular contractile
426 activity undoubtedly contributed given that physical activity acts synergistically to enhance
427 the muscle protein synthetic response to dietary protein/amino acids (24, 31, 32). In addition,
428 two weeks of step reduction has previously been shown to induce the development of
429 ‘anabolic resistance’ in older adults and thus it is possible that a similar phenomenon was
430 captured in the long-term measurement of myofibrillar protein synthesis rates in the present
431 study (10).

432 The precise acute metabolic mechanisms underpinning the step reduction-induced decline in
433 myofibrillar protein synthesis rates remain to be confirmed in a younger population.

434 Nonetheless, in the present study, a coordinated up-regulation of myostatin expression and
435 down-regulation of mTOR expression was observed in skeletal muscle following one week of
436 SR (**Figures 4C and 4D**). These findings are relatively consistent with previous studies that
437 have observed increased myostatin expression following human muscle disuse (33, 34).
438 Myostatin negatively regulates muscle mass in part via inhibition of the mechanistic target of
439 rapamycin (mTOR), a key regulator of muscle protein synthesis (35). Heightened mRNA
440 expression of myostatin in conjunction with lowered mRNA expression of mTOR is therefore
441 entirely consistent with the observed reduction in myofibrillar protein synthesis rates.

442 To gain further insight into the impact of short-term reduced physical activity and increased
443 sedentary time on muscle mass regulation, the gene expression of putative markers of muscle
444 protein breakdown was also determined. Muscle-specific E3 ubiquitin ligases (e.g., muscle
445 atrophy F-box (MAFbx) and muscle RING finger 1 (MuRF1)) selectively target muscle
446 proteins for degradation via the 26S proteasome (36). In the present study, an increase in
447 MAFbx expression was observed whereas MuRF1 expression remained unchanged (**Figures**
448 **4A and 4B**). The disparity in the responsiveness of the E3 ubiquitin ligases to step reduction
449 is intriguing but has been reported previously following bed rest and limb immobilization in
450 humans (13, 34). It is possible that the observed decrease in myofibrillar protein synthesis
451 rates was matched by a similar decrease in muscle protein breakdown, reflecting a reduced
452 muscle protein turnover, although the increase in MAFbx expression following step reduction
453 does not support this notion. However, this observation represents a single time point and
454 may not necessarily reflect dynamic changes that occurred throughout the entire step
455 reduction period. Clearly further research is required to provide greater insight into the
456 relative importance of muscle protein breakdown in the context of reduced physical activity
457 and increased sedentary time.

458 In line with previous findings, one week of reduced physical activity and increased sedentary
459 time led to a decline in whole-body insulin sensitivity (**Figure 2**). The increased plasma
460 insulin response (**Figures 2C and 2D**), without a significant change in the plasma glucose
461 response to the OGTT (**Figure 2A and 2B**), supports previous findings in young individuals
462 (37) and likely represents a compensatory mechanism in order to maintain glycaemic control.
463 This is in contrast to longer-term (2 weeks) step reduction, where both plasma glucose and
464 insulin concentrations appear to be elevated in response to an OGTT (11).

465 The absence of muscle mass measures following step reduction may be considered a
466 limitation of the present investigation. However, recent evidence has shown that myofibrillar
467 protein synthesis rates measured using $^2\text{H}_2\text{O}$ are predictive of long-term changes in skeletal
468 muscle mass (38). Thus, it is possible that the observed decline in daily myofibrillar protein
469 synthesis would contribute to loss of muscle mass with chronic reduced physical activity and
470 increased sedentary time. It should also be noted that structured physical activity was reduced
471 *and* sedentary time was increased in the present study, precluding any conclusions being
472 made on the independent impact of either of these distinct behaviours. However, given that a
473 large proportion of the global population are both physically inactive and highly sedentary
474 (1), the present findings are highly relevant. Finally, physical activity levels tend to be lower
475 in women compared to men and thus future research utilising a similar study design in
476 women is warranted (17).

477 In conclusion, one week of step reduction lowers daily myofibrillar protein synthesis rates
478 and alters the expression of several genes within skeletal muscle related to muscle mass
479 regulation and oxidative metabolism in healthy young men. Promotion of regular physical
480 activity and minimising sedentariness throughout the lifespan should be considered as
481 essential to the preservation of skeletal muscle health.

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490 **CONFLICTS OF INTEREST**

491 None of the authors have any conflicts of interest or financial disclosures to declare. The
492 results of the present study are presented clearly, honestly, and without fabrication,
493 falsification, or inappropriate data manipulation and do not constitute endorsement by the
494 American College of Sports Medicine.

495 **AUTHOR CONTRIBUTIONS**

496 B.J.S., L.J.C.v.L., J.L.T., and G.A.W. conception and design of research; B.J.S., Y.S.E., and
497 G.A.W. performed experiments; B.J.S., A.M.H., and B.S. analysed samples; B.J.S. and
498 G.A.W. prepared figures and drafted manuscript; B.J.S., A.M.H., B.S., Y.S.E., A.P.,
499 L.J.C.v.L., J.L.T., and G.A.W. edited and revised manuscript; B.J.S., A.M.H., B.S., Y.S.E.,
500 A.P., L.J.C.v.L., J.L.T., and G.A.W. approved final version of manuscript.

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664 **Table 1.** Participant characteristics at baseline

Variable	Value
Age (y)	22.2 ± 2.2
Height (m)	1.77 ± 0.08
Body mass (kg)	74.0 ± 11.0
BMI (kg·m ⁻²)	23.4 ± 2.4
Body fat (%)	18.6 ± 3.2
Whole body FFM (kg)	60.0 ± 7.2
Leg FFM (kg)	20.2 ± 2.4

665 Values are mean±SD. n=11. BMI, body mass index; FFM, fat-free mass.

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669 **Table 2.** Dietary intake during habitual physical activity (HPA) and step reduction (SR)

Variable	HPA	SR
Energy intake (kcal·d ⁻¹)	2625 ± 732	2380 ± 864
Protein (g·kg ⁻¹ ·d ⁻¹)	2.1 ± 0.7	1.8 ± 0.6*
Protein intake (g·d ⁻¹)	156 ± 51	133 ± 45*
Carbohydrate intake (g·d ⁻¹)	297 ± 142	279 ± 165
Fat intake (g·d ⁻¹)	83 ± 34	77 ± 33
Protein (En%)	26 ± 13	24 ± 12
Carbohydrate (En%)	46 ± 13	46 ± 12
Fat (En%)	28 ± 9	29 ± 10

670 Values are mean±SD. n=11. *(P<0.01) indicates a significant difference between HPA and SR

671 conditions.

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683 **FIGURE HEADINGS**

684 **Figure 1.** Study overview.

685 **Figure 2.** Plasma metabolite responses to an oral glucose tolerance test (OGTT) following 7
686 days of habitual physical activity (HPA) and 7 days of step reduction (SR) in young males
687 (n=11). Data are mean±SD. A two-factor repeated measures ANOVA was performed for
688 analysis of plasma glucose (A), insulin (C) and NEFA (E) responses to the OGTT. Plasma
689 glucose (B) and insulin (D) area under the curve (AUC) and Matsuda index (F) were
690 analysed using paired sample t-tests. A: significant main effect for time (P<0.001), *P<0.01
691 compared to all other time points, †P<0.05 compared to 0 and 120 minutes, ‡P<0.05
692 compared to 120 minutes. B: no significant effect. C: significant condition x time interaction
693 (P<0.01), *P<0.05 compared with corresponding HPA value. D: *P<0.01 compared with
694 HPA. E: significant main effect for time (P<0.001), *P<0.05 compared to all other time
695 points, †P<0.001 compared to 60, 90 and 120 minutes, ‡P<0.05 compared to 90 minutes. F:
696 *P<0.01 compared with corresponding HPA value.

697 **Figure 3.** Body water ²H enrichment and daily myofibrillar protein fractional synthesis rates
698 (FSR) during 7 days of habitual physical activity (HPA) and 7 days of step reduction (SR) in
699 young males (n=11). Data are mean±SD. Body water ²H enrichment (A) was analysed using
700 a one-factor repeated measures ANOVA. Myofibrillar protein FSR (B) was analysed using a
701 paired sample t-test. Body water ²H enrichment remained in steady state for the duration of
702 the study (P>0.05). *(P<0.05) indicates a significant difference between HPA and SR
703 conditions.

704 **Figure 4.** Skeletal muscle mRNA expression of muscle RING finger 1 (MuRF1; A) muscle
705 atrophy F-box (MAFbx; B), myostatin (C), the mechanistic target of rapamycin (mTOR; D),
706 ribosomal protein S6 kinase beta-1 (p70S6K; E), p53 (F), pyruvate dehydrogenase kinase 4

707 (PDK4; G) and peroxisome proliferator activated receptor gamma coactivator 1-alpha (PGC-
708 1 α ; H) following 7 days of habitual physical activity (HPA) and 7 days of step reduction (SR)
709 in young males (n=10). Data are mean \pm SD. Data were analysed using paired sample t-tests.

710 *(P<0.05) indicates a significant difference between HPA and SR conditions.

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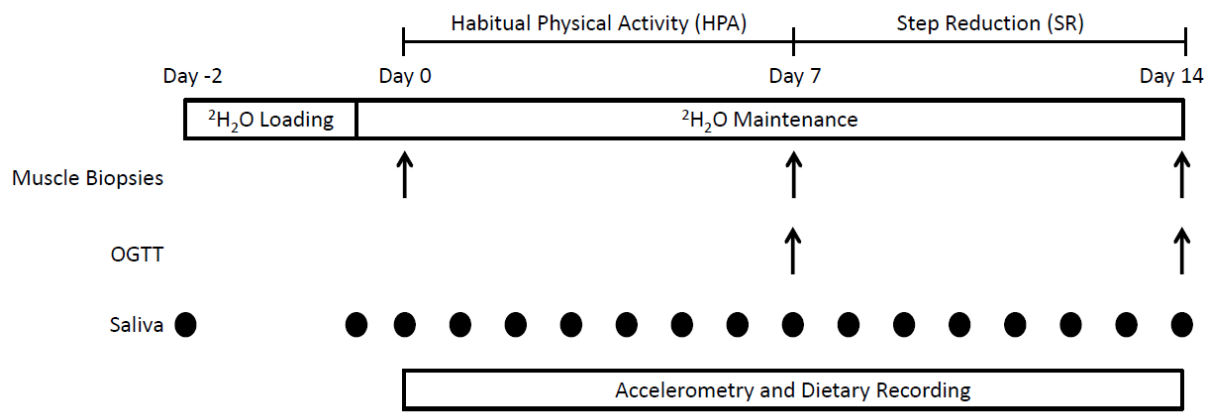
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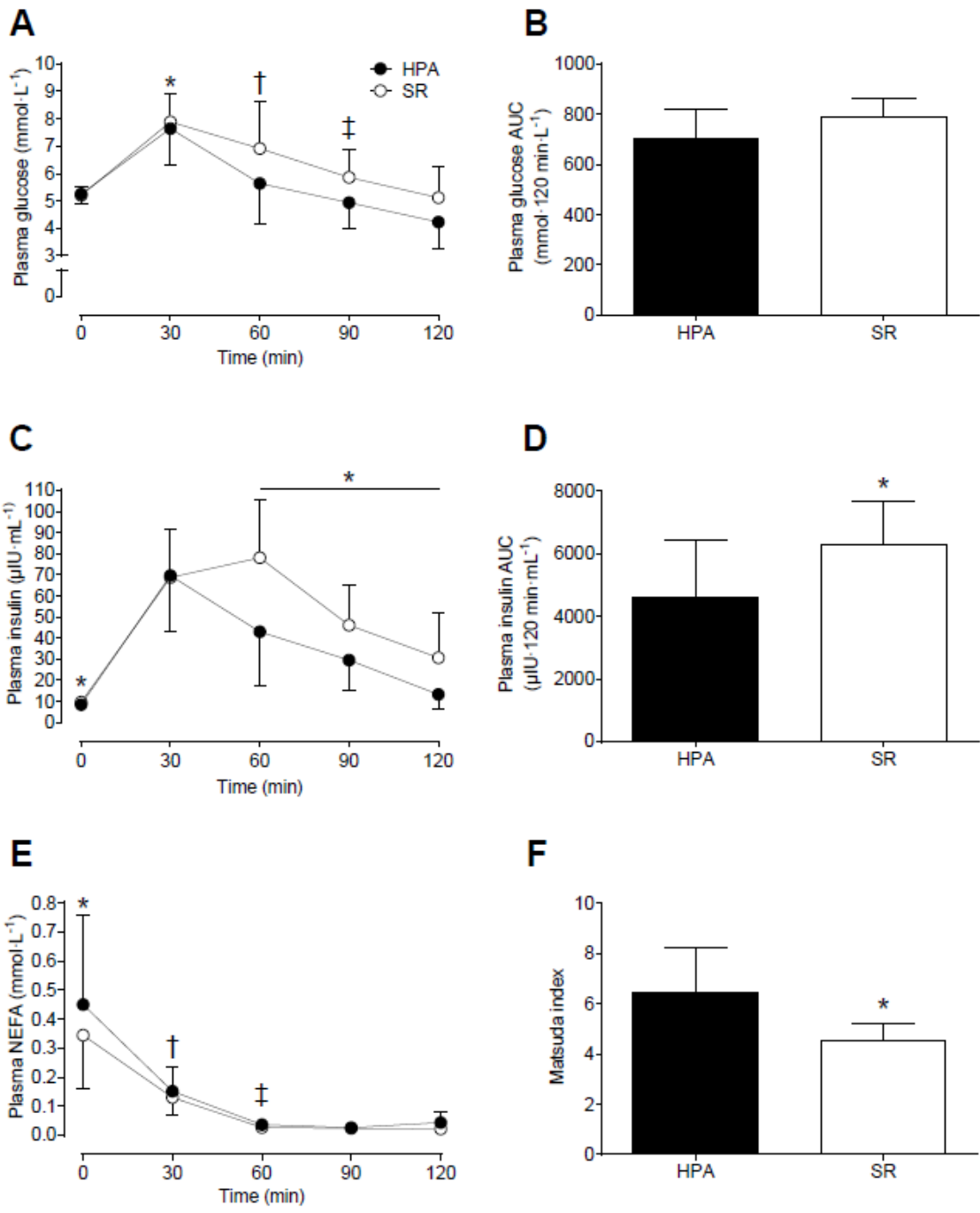
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730 **Figure 1.**

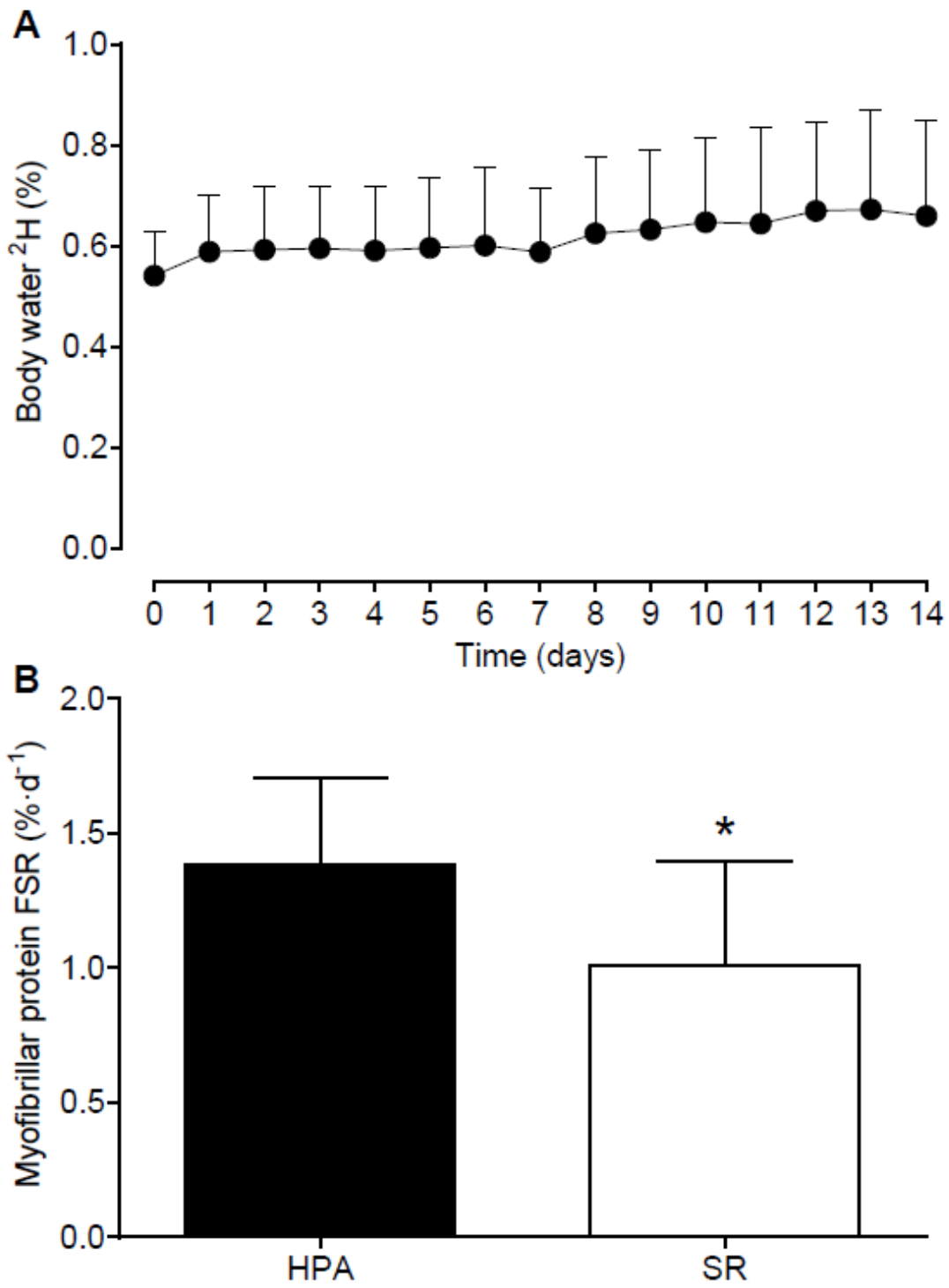


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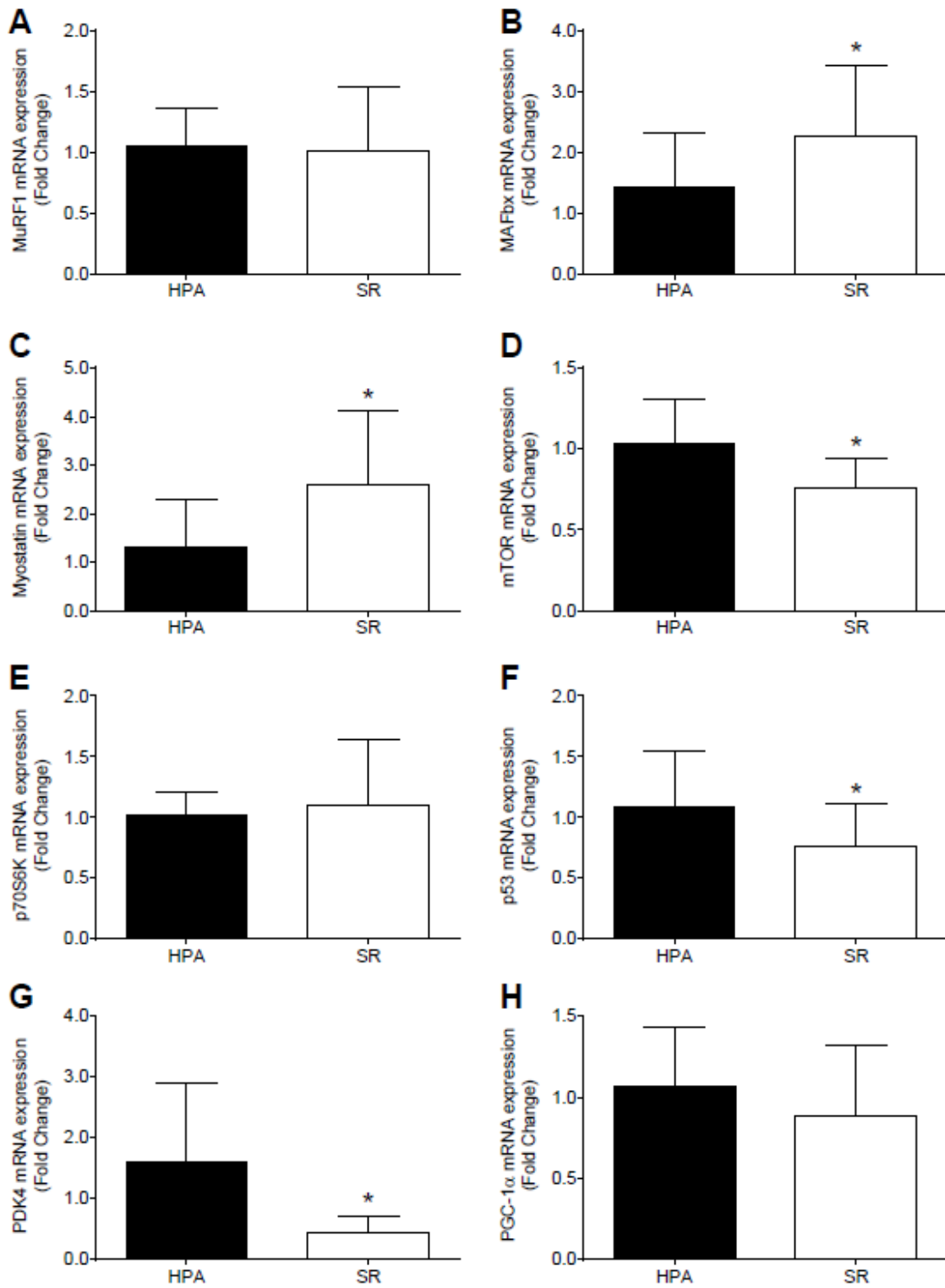


736 **Figure 3.**



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742 **Supplementary Table 1. RT-qPCR primer sequences**

Gene	Forward primer	Reverse primer
MuRF1	5'-GACGCCCTGAGAGCCATT-3'	5'-CCTCTTCCTGATCTTCTTCAAT-3'
MAFbx	5'-AACTCAAATACAAAATAGGACGCTTT-3'	5'-CCTTCGCCTTCTCAAAACAAAC-3'
Myostatin	5'-GTCGAGACTCCTACAACAGTG-3'	5'-TCCAGTATACCTTGTACCGTCTT-3'
mTOR	5'-CTGATGCTGGACCGTCTGA-3'	5'-TCTTGTTAGTCTAAATGGAATCTTCTC-3'
p70S6K	5'-GCAAGCTGGACAAACTATCACA-3'	5'-CCACTGAGATAATACTTGTGCTATAATG-3'
p53	5'-GTGGAGTATTTGGATGACAGAAAC-3'	5'-GTAGTTGTAGTGGATGGTGGTAC-3'
PDK4	5'-GAGGGACTCAGGACACTTTAC-3'	5'-TGGAGGAAACAAGGGTTCACAC-3'
PGC-1 α	5'-TTGCTAAACGACTCCGAGAAC-3'	5'-GACCCAAACATCATACCCCAAT-3'

743 MuRF1, muscle RING finger 1; MAFbx, muscle atrophy F-box; mTOR, the mechanistic target of rapamycin;
 744 p70S6K, ribosomal protein S6 kinase beta-1; PDK4, pyruvate dehydrogenase kinase 4; PGC-1 α , peroxisome
 745 proliferator activated receptor gamma coactivator 1-alpha.